Dietary protein and zinc restrictions independently modify a *Heligmosomoides polygyrus* (Nematoda) infection in mice

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SUMMARY

The effects of dietary protein and zinc restrictions on *Heligmosomoides polygyrus* were compared following primary and challenge infection in female BALB/c mice fed either control (24 %), marginal (7 %) or low (3 %) protein combined with either high or low zinc (60 or 3 mg Zn/kg diet). Dietary protein restriction (3 %) resulted in significantly lower body weight gain. As well, blood urea nitrogen (BUN) significantly decreased with decreasing dietary protein level. However, neither plasma albumin concentration nor relative thymus or spleen weights were reduced. Marginal zinc deficiency was confirmed by significantly lower tibia and liver zinc concentration, but food intake, body weight gain, relative thymus and spleen weights, and alkaline phosphatase activity were not altered. On day 29 post-primary infection, worm burdens were significantly higher in mice fed either marginal or low protein and in mice fed a low zinc diet, while parasite egg output was significantly higher in mice fed both low protein and low zinc diets. Immune status was compromised in mice fed low protein (significantly lower serum IgG1 and lower eosinophilia), and in mice fed low zinc diet (significantly lower eosinophilia). Early in the infection, IgE titres were elevated in mice fed low protein or low zinc, but IgE titres declined to levels lower than the control diet groups after 14–21 days. On day 29 post-challenge infection, worm burdens and parasite egg output were significantly higher in mice fed low protein, whereas the other groups had expelled almost all parasites. Dietary restriction had no effect on serum IgE. Significantly reduced serum IgG1 titres and eosinophilia in mice fed 3 % protein supported the view that low dietary protein but not low zinc increased host susceptibility to *H. polygyrus* by compromising host immune function following reinfection in immunized mice.

Key words: *Heligmosomoides polygyrus*, zinc restriction, protein deficiency, egg production, survival, antibody response, eosinophilia.

INTRODUCTION

Malnutrition and gastrointestinal nematode infections are chronic diseases that often co-exist in individuals in developing countries (Pawlowski, 1984). Host malnutrition is believed to alter the rate of parasite transmission and the intensity of infection by interfering with host immune function or by interfering directly with the intestinal environment and the nutritional needs of the parasite (Bundy & Golden, 1987). Because of the association between either protein or zinc and host immune function (Gershwin, Beach & Hurley, 1985; Chandra, 1988), the effects of isolated protein and zinc deficiencies have been studied in experimental animal models of human intestinal nematode infections.

Protein deficiency has been shown to delay expulsion of *Nippostrongylus brasiliensis* in rats (Bolin *et al*. 1977; Duncombe, Bolin & Davis, 1979), and *Trichuris muris* in mice (Michael & Bundy, 1991), and to prolong *Heligmosomoides polygyrus* survival and increase its fecundity in mice, both after repeated infections (Slater & Keymer, 1986*a*; Keymer & Tarlton, 1991) and during continuous natural transmission of the parasite (Slater & Keymer, 1986*b*; Slater, 1988). Protein deficiency also impaired the ability of gamma-irradiated larvae during a challenge infection protocol to immunize mice against *H. polygyrus* re-infection (Slater & Keymer, 1988). In contrast to this, Brailsford & Mapes (1987) found that protein deficiency had no effect on *H. polygyrus* establishment or survival during a primary infection.

The effects of zinc deficiency on intestinal nematode infections have been less consistently demonstrated. In a study by El-Hag *et al*. (1989), no effect of reduced dietary zinc (3 mg Zn/kg diet) was found on the number or size of *N. brasiliensis* in rats. In contrast, Fenwick *et al*. (1990) reported delayed expulsion of *Trichinella spiralis* and higher worm burdens in rats fed a zinc deficient diet (3 mg Zn/kg diet) compared to control animals. In our laboratory,

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a moderately low dietary zinc intake (5 mg Zn/kg diet) had no effect on *H. polygyrus* establishment or fecundity in mice (Minkus, Koski & Scott, 1992), whereas a more severe zinc deficiency (0·75 mg Zn/kg diet) prolonged parasite survival during both primary and challenge *H. polygyrus* infections (Shi *et al*. 1995).

No systematic study has been undertaken previously to examine the effects of combined protein and zinc deficiency, even though the combination of the 2 dietary deficiencies has particular relevance to the 'real world' where they often co-exist (Fraker *et al*. 1986). The purpose of this study was to determine whether a combined dietary protein and zinc deficiency would have independent or interacting effects on the outcome of primary and challenge infections of mice with *H. polygyrus* in mice. *H. polygyrus* is a trichostrongyloid nematode used widely as an experimental model for studying host immunology, immunogenetics and epidemiology of chronic gastrointestinal nematodes of humans and livestock (Monroy & Enriquez, 1992; Scott & Tanguay, 1994). Primary infections with *H. polygyrus* persist for several weeks or months (Ehrenford, 1954), whereas challenge infections elicit strong and rapid protective immunity, manifested by reduced parasite reproduction and increased expulsion of adult worms (Behnke & Robinson, 1985; Scott, 1991), elevated IgG1 and IgE titres and eosinophilia (Hurley & Vadas, 1983; Pritchard *et al*. 1983; Urban, Katona & Finkelman, 1991). The specific objectives were to investigate potential synergistic effects of marginal and low protein restriction combined with zinc restriction on parasite establishment, survival and reproduction, and on antibody (IgG1 and IgE) and eosinophil responses to *H. polygyrus*.

MATERIALS AND METHODS

Experimental design

A 3×2 factorial design that combined 3 levels of dietary protein (24 %–control, 7 %–marginal and 3 %–low) with 2 levels of dietary zinc (60 mg zinc/kg diet–control and 3 mg zinc/kg diet–low) was used during both a primary infection and a challenge infection protocol.

Animals and housing

All procedures were conducted in conformance with guidelines for experimental procedures set forth by McGill's local animal care committee and by the Canadian Council on Animal Care (1984). Weanling (21 day), inbred BALB/c female mice (Charles River, St Constant, Quebec) were individually housed in Nalgene cages ($27 \times 21 \times 14$ cm) having stainless steel covers, and stainless steel floor grids (Fisher Scientific, Montreal, Quebec) to prevent coprophagy. The diets were dispensed in plastic mouse powder feeders (Lab Products Inc., Montreal, Quebec) specifically designed to minimize food spillage. Deionized water was freely available from Nalgene bottles with Neoprene stoppers (Fisher Scientific). Cages, stainless steel covers and grids, bottles and feeders were all acid-washed prior to use and plastic filter tops were placed over the cages to minimize environmental zinc contamination. Room temperature was maintained between 22 and 25 °C and lighting was on a 14 h light : 10 h dark cycle.

Parasite

Infective 3rd-stage larvae (L_3) of *H. polygyrus* were obtained by culturing the faeces of chronically infected stock CD1 mice (Charles River, St Constant, Quebec) on moist filter paper for 7 days. The cultured larvae were administered to mice in 0·02 ml deionized water using a Pipetteman with soft plastic tips. The accuracy of the inoculum was estimated by direct counts of the number of larvae in each of 5 sham doses which were dispensed into plastic Petri dishes before the experimental infection.

Diets

All mice were fed a semi-purified biotin-fortified diet with spray-dried egg white solids as the sole source of protein (Table 1). Three dietary protein levels were chosen. The 24 % control level is considered adequate for most strains of mice and not excessive for any (NRC, 1978). The protein-restricted diets contained either 7 % (marginal) or 3 % (low) protein and were made isoenergetic to the control diet (24 % protein) on a weight basis by replacing an amount of protein in the deficient diets with an equal quantity of starch; all other dietary constituents were identical. Zinc was added as zinc carbonate (Fisher Scientific, Montreal, Quebec) to provide 60 mg/kg to the control diet and 3 mg/kg to the zinc deficient diet; dietary levels were verified by flame atomic absorption spectrophotometry (Perkin–Elmer 3100, Perkin–Elmer Canada Ltd, Montreal, Quebec). The 60 mg zinc/kg diet was twice the level recommended by NRC (1978). This higher level was chosen to ensure adequate intakes should food intake be reduced by 50 %, which is a common occurrence when protein-deficient diets are fed.

Experimental protocol

Mice were acclimatized by feeding the control diet (24 % protein, 60 mg zinc/kg diet) for 3 days before being randomly assigned to the 6 dietary treatments. Food intake (corrected for spillage) and body weights were measured every second day. On day 22 of the

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Table 1. Composition of the control diet (24 % protein)

Ingredient	g/kg
Egg white solids	240
Cornstarch	296
Glucose	296
Corn oil	80
Cellulose	30
Vitamin mix*	12
Mineral mix†	46

* Vitamin mix provided the following (mg/kg diet): niacin 50·0; calcium pantothenate 32·0; riboflavin 24·0; pyridoxine hydrochloride 4·0; thiamine hydrochloride 16·0; folacin 2·0; biotin 3·6; cyanocobalamine 0·05; alpha-tocopheryl acetate 45·5; menaquinone 9·0; cholecalciferol 0·975; retinyl palmitate 7·6; choline chloride 4000.
† Mineral mixture provided the following (g/kg diet): $CaHPO_4$ 27·16; $KHCO_3$ 10·25; NaCl 2·54; $MgSO_4$ 4·95; $CrK(SO_4)_2$ 0·0157; $KClO_3$ 0·001; $FeSO_4.7H_2O$ 0·2489; $MnCO_3$ 0·1883; $NaSeO_3$ 0·0004; $NaMoO_4.2H_2O$ 0·0002; $KF.2H_2O$ 0·0099.

dietary treatment, 11 mice/dietary group were given a first infection with 100 L_3 (day 0 post-primary infection). Of these mice, 3/group served as infection controls to verify the establishment of the parasite and were killed on day 9 post-primary infection. The other 8 mice/diet group were treated with 175 mg/kg pyrantel pamoate (Combantrin, Pfizer, Montreal, Quebec) on day 9 and on day 14 post-primary infection, to remove established *H. polygyrus*. One week later, 3 mice/diet group were killed as drug controls. As has been the case in all our previous studies, no worms were found in the intestines of these drug controls, thus confirming the efficacy of the drug treatment. On the same day (day 21 post-primary infection and now day 0 post-challenge) the remaining 5 mice/group were re-infected with 100 L_3 (challenge infection group). At the same time, 5 naive mice/dietary group were infected for the first time with 100 L_3 (primary infection group).

In both the primary and challenge-infected mice, blood was obtained from the tail vein on day 0 post-infection for baseline immunoglobulin measurement, and on days 7, 14, 21 and 29 for the quantification of blood eosinophils and serum IgG1. Twenty-four h stool collections were made on days 16, 22 and 28 post-infection for the determination of daily parasite egg output. On day 29 post-infection, mice were anaesthetized with Metofane (CDMV Inc., Jansse, Quebec, Canada); blood was collected using heparinized capillary tubes, and was immediately transferred to heparinized, acid-washed 1·5 ml polyethylene Eppendorf tubes which were centrifuged to obtain plasma. Plasma was immediately separated and transferred into acid-washed Eppendorf tubes, placed on dry ice and stored at −80 °C for future determination of plasma albumin, BUN, and alkaline phosphatase concentrations. Serum separator tubes (Microtainer Brand Serum Separator Tubes, Becton-Dickenson, Fisher Scientific, Montreal, Quebec) containing serum were frozen at −20 °C for later determination of IgG1. Mice were killed by cervical dislocation. The liver, spleen and thymus were cleaned of adhering tissue, and weighed. The entire left posterior leg was removed and placed in polyethylene Eppendorf tubes for later zinc extraction from tibia bone. All tubes containing tissues were immediately put on dry ice and stored at −80 °C. The small intestine was removed and placed in plastic bags and stored at −20 °C for later determination of worm numbers.

Nutrition assays

Albumin concentration was determined by dye-binding of bromcresol green at pH 4·2 (Albumin procedure No. 631, Sigma Diagnostics, St Louis, MO) based on the procedure of Doumas, Watson & Biggs (1971). BUN concentration was determined by coupled enzyme reactions involving urease and glutamate dehydrogenase (BUN Procedure No. 67-uv, Sigma Diagnostics, St Louis, MO) based on the procedure of Talke & Schubert (1965). Alkaline phosphatase activity was determined by the hydrolysis of *p*-nitrophenyl phosphate (Alkaline phosphatase Procedure No. 245, Sigma Diagnostics, St Louis, MO) based on the procedure of Bowers & McComb (1966). Analyses were performed on an Abbott VP Super System Discrete Analyzer (Abbott Diagnostic) immediately after thawing of plasma.

Tibia were analysed for zinc, and livers for zinc and copper by flame atomic absorption spectrophotometry (Perkin–Elmer 3100). Tissues were freeze-dried for at least 24 h prior to wet ashing with nitric acid according to the method of Clegg *et al.* (1981). Liver samples were fat extracted with petroleum ether using a Soxtec System HT2. A blank containing nitric acid only and National Bureau of Standards Reference Material (Bovine Liver 1577a) were digested and analysed with the samples.

Parasitological measures

Daily egg output of *H. polygyrus* was estimated from complete 24 h stool collections by a modified McMaster technique (Scott, 1988). Egg counts for each mouse were expressed as total eggs/mouse/day on days 16, 22 and 28 post-infection. The gender and number of adult *H. polygyrus* were determined by examining thawed intestines that were slit open onto a glass plate and scanned using a dissection microscope. The number of 4th-stage larvae (L_4) of *H. polygyrus* remaining in the serosal musculature was also recorded in infection controls on day 9 post-primary infection.

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Immunology assays

Eosinophils were counted with the UNOPETTE Test 5877 (Fisher Scientific). Two Neubauer haemacytometer chambers were counted for each blood sample, and the average was recorded. The level of total IgG1 in serum was determined by an enzyme-linked immunosorbent assay (ELISA). Immulon II plates (Dynatech Laboratories, Inc., Chantilly, VA) were coated with serial dilutions of purified mouse IgG1 Kappa (MOPC-31c) immunoglobulin (Sigma Immuno Chemicals, St Louis, MO) together with diluted serum samples, and incubated overnight at 4 °C. Peroxidase-conjugated rabbit anti-mouse IgG1 monoclonal antibody (Serotec, UK) was diluted as a detecting antibody (1:1000). The reaction was visualized with the Bio-Rad Peroxidase substrate kit ABTS (Bio-Rad, Hercules, CA) at 415 nm on an ELISA reader.

The level of parasite-specific IgG1 in serum was determined by an indirect ELISA. Immulon II plates were coated with 4th-stage larvae (L_4) *H. polygyrus* antigen (preparation described below) at 2·5 μl and incubated overnight at 4 °C. Serum samples, along with infected and naive control sera, were added in duplicate at a dilution of 1:500 and incubated overnight at 4 °C. Peroxidase-conjugated rabbit anti-mouse IgG1 (Serotec, UK) was diluted as a detecting antibody. The reaction was visualized with ABTS substrate solution (Sigma, St Louis, MO). Parasite antigen was prepared using L_4 *H. polygyrus* obtained from the intestine of mice 6 days post-infection (Shi *et al.* 1994). Parasites were homogenized on ice in phosphate buffer solution. The homogenate was centrifuged at 1500 ***g*** at 4 °C for 1 h and the supernatant recovered and sterilized using a 0·2 μm Acrodisc (Gelman Sciences, Ann Arbor, MI). Protein concentration was determined using a commercially available assay (Bio-Rad, Mississauga, Ontario, Canada).

The level of total IgE in serum was determined by a sandwich ELISA. Briefly, Immulon II plates were coated with purified rat anti-mouse IgE mAb (2 μg/ml) (R35-72, PharMingen, San Diego, CA), and incubated at 4 °C overnight. Alkaline phosphatase-conjugated rat anti-mouse IgE mAb (R35-118, PharMingen) was used as a detecting Ab (1:2000). The reaction was visualized with *p*-nitrophenyl-phosphate (PNPP) (Bio-Rad Laboratories, Richmond, CA) (1 mg/ml).

Statistical analyses

Since 2 different infection protocols were used (primary and challenge), the data for the nutritional parameters were first analysed with a 3-way ANOVA including as main effects in the statistical model: infection protocol, protein, zinc and their interactions. If differences were detected between infection protocols, the data were analysed separately by infection protocols using a two-way ANOVA that included the main effects of protein, zinc and their interactions (zinc × time, protein × time) and data were reported by infection protocol. This was done for relative liver weight, plasma albumin, alkaline phosphatase activity, and tibia and liver zinc concentrations. Cumulative food intake, body, thymus and spleen weights, BUN and liver copper concentration were not affected by the infection protocol. Therefore, the combined data (primary + challenge infection protocols and their means) were reported for these nutritional status parameters.

The parasitological and immunological data were analysed for each infection protocol using the above model or by a 3-way ANOVA including the effect of time (and all interactions) when appropriate. Due to the fact that parasite data are often not normally distributed, all parasite and immunological parameters were first tested for normality with the Shapiro-Wilk statistic W. The residual was tested for normality, rather than the specific variable itself, to minimize treatment effects on the distribution. When appropriate, a square root (SQRT) transformation was used, resulting in all data meeting the assumption of normality.

Since food intake was significantly greater in mice fed 7 % protein as compared to other protein levels, food intake was included in the statistical model as a covariate. If the main dietary effects of zinc and/or protein were significant, this was followed by individual *post-hoc* multiple pairwise comparisons (Tukey's test), which established exactly which dietary levels differed from one another. All statistical analyses were done with the SAS program (SAS Institute Inc., 1985) and the level of significance was set at $\alpha = 0{\cdot}05$.

RESULTS

Food intake, body weight and tissue weights

In both a primary and challenge infection (Table 2), there was a significant effect of dietary protein ($P = 0{\cdot}0001$), but not zinc, on cumulative food intake and body weight. Mice fed 7 % protein ate more food than mice fed 24 % and 3 % protein. Mice fed 3 % protein had significantly lower final body weight and lower body weight gain (data not shown) than mice fed either 7 % or 24 % protein. In both the primary and challenge infections (Table 2), dietary protein and zinc had no effect on relative thymus weight. Relative spleen weight was affected by dietary protein ($P = 0{\cdot}04$), but not dietary zinc, such that mice fed 7 % protein had higher relative spleen weights than mice fed 24 % and 3 % protein. In a primary infection, dietary zinc ($P = 0{\cdot}008$), but not protein, altered relative liver weight, with mice fed 3 mg Zn/kg diet having higher values than mice fed 60 mg Zn/kg diet (Table 3). In a challenge infection,

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Table 2. The effect of dietary protein and zinc restrictions on nutritional parameters of mice that did not differ by infection protocol on day 29 post-infection

(Values are means ± S.E.M.; the data for the primary and challenge infections are pooled; the number of animals in each group is 10. Values within each parameter with different letter superscripts are statistically different ($P \leqslant 0{\cdot}05$) based on Tukey's multiple *post-hoc* comparisons.)

	Protein level (%)			Zinc (mg/kg)	
Parameter	24	7	3	60	3
Total food intake (g)†	$215{\cdot}7 \pm 2{\cdot}4^{b}$	$242{\cdot}8 \pm 2{\cdot}5^{a}$	$205{\cdot}8 \pm 4{\cdot}5^{b}$	$221{\cdot}7 \pm 4{\cdot}0$	$221{\cdot}2 \pm 3{\cdot}8$
Final body weight (g)†	$20{\cdot}6 \pm 0{\cdot}3^{a}$	$21{\cdot}0 \pm 0{\cdot}3^{a}$	$14{\cdot}6 \pm 0{\cdot}4^{b}$	$19{\cdot}1 \pm 0{\cdot}6$	$18{\cdot}4 \pm 0{\cdot}6$
Organ weights (% bwt)					
Spleen‡	$0{\cdot}43 \pm 0{\cdot}001^{b}$	$0{\cdot}50 \pm 0{\cdot}02^{a}$	$0{\cdot}39 \pm 0{\cdot}02^{b}$	$0{\cdot}43 \pm 0{\cdot}02$	$0{\cdot}45 \pm 0{\cdot}02$
Thymus§	$0{\cdot}22 \pm 0{\cdot}01$	$0{\cdot}20 \pm 0{\cdot}01$	$0{\cdot}20 \pm 0{\cdot}01$	$0{\cdot}21 \pm 0{\cdot}01$	$0{\cdot}20 \pm 0{\cdot}01$
Plasma					
BUN (mg/kg)†	$22{\cdot}4 \pm 0{\cdot}9^{a}$	$16{\cdot}5 \pm 0{\cdot}5^{b}$	$11{\cdot}3 \pm 0{\cdot}6^{c}$	$16{\cdot}7 \pm 1{\cdot}1$	$17{\cdot}2 \pm 0{\cdot}9$
Tissue					
Liver copper (mg/g)‡	$18{\cdot}8 \pm 0{\cdot}5^{b}$	$18{\cdot}7 \pm 0{\cdot}5^{b}$	$20{\cdot}7 \pm 0{\cdot}9^{a}$	$20{\cdot}1 \pm 0{\cdot}9$	$18{\cdot}7 \pm 0{\cdot}4$

Significance of main effects from 2-way AOV.
† Protein ($P < 0{\cdot}0001$); zinc (N.S.); zinc × protein (N.S.).
‡ Protein ($P = 0{\cdot}04$); zinc (N.S.); zinc × protein (N.S.).
§ Protein (N.S.); zinc (N.S.); zinc × protein (N.S.).

Table 3. The effect of dietary protein and zinc restrictions on nutritional parameters of mice on day 29 post-infection where data differed by infection protocol

(Values are means ± S.E.M.; the number of animals in each group is 5. Values within each parameter with different letter superscripts are statistically different ($P \leqslant 0{\cdot}05$) based on Tukey's *post-hoc* comparisons when interaction is NS.)

	Protein level (%)			Zinc (mg/kg)	
Parameter	24	7	3	60	3
			PRIMARY		
Relative liver wt (% bwt)†	$4{\cdot}14 \pm 0{\cdot}12$	$4{\cdot}20 \pm 0{\cdot}09$	$4{\cdot}29 \pm 0{\cdot}10$	$4{\cdot}06 \pm 0{\cdot}08^{b}$	$4{\cdot}38 \pm 0{\cdot}07^{a}$
Plasma					
Albumin (g/dl)‡	$2{\cdot}97 \pm 0{\cdot}1$	$3{\cdot}11 \pm 0{\cdot}1$	$3{\cdot}25 \pm 0{\cdot}1$	$2{\cdot}90 \pm 0{\cdot}1^{b}$	$3{\cdot}33 \pm 0{\cdot}1^{a}$
Alkaline phosphatase§ (I.U.)	$99{\cdot}6 \pm 5{\cdot}2$	$100{\cdot}9 \pm 4{\cdot}3$	$184{\cdot}1 \pm 8{\cdot}2$	$116{\cdot}5 \pm 9{\cdot}9$	$153{\cdot}4 \pm 12{\cdot}7$
Tissue					
Tibia zinc (μg/kg)‖	198 ± 10^{a}	196 ± 14^{a}	148 ± 6^{b}	205 ± 9^{a}	153 ± 5^{b}
Liver zinc (μg/kg)¶	118 ± 3^{a}	114 ± 2^{b}	107 ± 3^{b}	118 ± 2^{a}	108 ± 2^{b}
			CHALLENGE		
Relative liver wt (% bwt)††	$4{\cdot}20 \pm 0{\cdot}11$	$4{\cdot}32 \pm 0{\cdot}11$	$4{\cdot}81 \pm 0{\cdot}15$	$4{\cdot}58 \pm 0{\cdot}14$	$4{\cdot}32 \pm 0{\cdot}14$
Plasma††					
Albumin (g/dl)‡‡	$2{\cdot}72 \pm 0{\cdot}1$	$3{\cdot}03 \pm 0{\cdot}07$	$2{\cdot}90 \pm 0{\cdot}11$	$3{\cdot}00 \pm 0{\cdot}1^{a}$	$2{\cdot}75 \pm 0{\cdot}1^{b}$
Alkaline phosphatase§§ (I.U.)	$99{\cdot}5 \pm 3{\cdot}7^{b}$	$94{\cdot}6 \pm 5{\cdot}6^{b}$	$147{\cdot}5 \pm 13{\cdot}6^{a}$	214 ± 8	179 ± 6
Tissue					
Tibia zinc (μg/kg)‖‖	201 ± 7^{a}	217 ± 12^{a}	171 ± 5^{b}	214 ± 8^{a}	179 ± 6^{b}
Liver zinc (μg/kg)¶¶	112 ± 1	116 ± 3	96 ± 3	110 ± 2	106 ± 4

Significance of main effects from 2-way AOV.
† Protein (N.S.); zinc ($P = 0{\cdot}008$); zinc × protein (N.S.).
‡ Protein (N.S.); zinc ($P < 0{\cdot}0001$); zinc × protein (N.S.).
§ Protein ($P < 0{\cdot}0001$); zinc ($P = 0{\cdot}001$); zinc × protein ($P = 0{\cdot}03$).
‖ Protein ($P < 0{\cdot}0001$); zinc ($P = \leqslant 0{\cdot}0001$); zinc × protein (N.S.).
¶ Protein ($P = 0{\cdot}02$); zinc ($P < 0{\cdot}0001$); zinc × protein (N.S.).
†† Protein ($P = 0{\cdot}002$); zinc ($P = 0{\cdot}04$); zinc × protein ($P = 0{\cdot}01$).
‡‡ Protein (N.S.); zinc ($P = 0{\cdot}01$); zinc × protein (N.S.).
§§ Protein ($P = 0{\cdot}001$); zinc (N.S.); zinc × protein ($P = 0{\cdot}07$).
‖‖ Protein ($P = 0{\cdot}005$); zinc ($P < 0{\cdot}0001$); zinc × protein (N.S.).
¶¶ Protein ($P < 0{\cdot}0001$); zinc (N.S.); zinc × protein ($P = 0{\cdot}007$).

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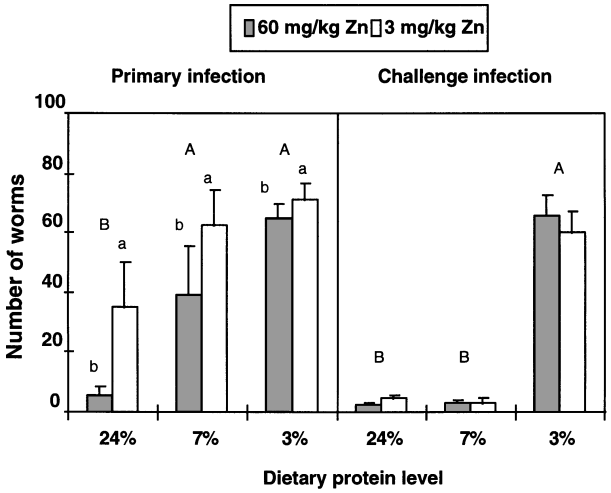

Fig. 1. The effects of dietary protein and zinc restrictions on worm burden on day 29 post-infection. Values are means ± S.E.M. $N = 5$ mice/dietary treatment and infection protocol. Significant differences for dietary protein for each infection protocol are denoted with different capital letters. Significant differences between the 2 levels of dietary zinc are denoted by different lower-case letters.

the relative liver weight was affected by a significant interaction between levels of dietary protein and zinc ($P = 0{\cdot}01$). Mice fed 60 mg Zn/kg diet and 3 % protein had higher relative liver weights than all the other groups (Table 3).

Plasma analyses

In a primary infection (Table 3), albumin was altered by the level of dietary zinc ($P = 0{\cdot}0001$), but not dietary protein. Mice fed 3 mg Zn/kg diet had higher plasma albumin than mice fed 60 mg Zn/kg diet. Alkaline phosphatase activity was affected by a significant interaction between dietary protein and zinc levels ($P = 0{\cdot}03$), such that alkaline phosphatase activity was highest in mice fed the 3 % protein combined with 3 mg Zn/kg diet.

In a challenge infection (Table 3), zinc restriction ($P = 0{\cdot}01$), but not protein restriction, produced significantly lower albumin values. On the other hand, alkaline phosphatase activity was affected by the level of dietary protein ($P = 0{\cdot}001$), but not by the level of dietary zinc. Alkaline phosphatase activity was significantly higher in mice fed 3 % protein than mice fed higher levels of dietary protein.

In both a primary and challenge infection (Table 2), BUN was significantly affected by protein level ($P = 0{\cdot}0001$), such that BUN decreased significantly with decreasing dietary protein levels. There was no effect of dietary zinc on BUN concentration.

Tissue trace elements

In both infection protocols, tibia zinc concentration was significantly lower in mice fed 3 % protein compared to either the 7 or 24 % protein groups ($P = 0{\cdot}0001$ in a primary infection and $P = 0{\cdot}005$ in a challenge infection), and it was further decreased by zinc restriction ($P = 0{\cdot}0001$ in both infection protocols) such that mice fed 3 mg Zn/kg diet had lower tibia zinc than mice fed 60 mg Zn/kg diet for each level of dietary protein intake. Liver zinc concentration was also modified by both dietary restrictions in the primary and challenge infection protocols (Table 3). In a primary infection, mice fed 3 % protein had lower liver zinc than mice fed 24 % protein ($P = 0{\cdot}02$), and mice fed 3 mg Zn/kg diet had lower liver zinc than mice fed 60 mg Zn/kg diet ($P = 0{\cdot}0001$). However, in a challenge infection, there was an interaction between dietary protein and zinc ($P = 0{\cdot}007$) such that the zinc concentration was lower only in mice fed the 3 % protein combined with 3 mg/kg zinc diet. Liver copper concentration was not affected by infection protocol or by zinc restriction (Table 2) but was significantly higher in mice fed the low 3 % protein diet compared to higher protein levels ($P = 0{\cdot}04$).

Worm burdens and worm egg production

The result from the infection control mice killed on day 9 post-primary infection indicated that 53–83 % of the L_3 parasite established in all groups, and, although the 3 % protein groups had significantly higher parasite establishment than the 7 % protein groups, neither differed from mice fed the control diet. Very few L_4 remained in the intestinal mucosa, and numbers did not differ among the groups (data not shown).

On day 29 post-primary infection (Fig. 1), mice fed either low (3 %) or marginal (7 %) levels of dietary protein had significantly more worms than mice fed adequate (24 %) protein ($P = 0{\cdot}001$), and mice fed low zinc (3 mg Zn/kg) diet had significantly more worms than mice fed a high zinc (60 mg Zn/kg) diet ($P = 0{\cdot}04$). In contrast to the primary infection group, the level of zinc in the diet had no effect on worm burdens, day 29 post-challenge infection. However, mice fed a low (3 %) protein diet had significantly higher worm burdens than mice fed 7 and 24 % protein, which were not different ($P = 0{\cdot}0001$).

In a primary infection (Fig. 2A), total daily parasite egg production was affected by dietary protein ($P = 0{\cdot}001$) and by dietary zinc ($P = 0{\cdot}02$). However, there was also significant protein and zinc interaction ($P = 0{\cdot}009$) and significant protein and time interaction ($P = 0{\cdot}009$). The nature of these interactions is displayed in Fig. 2B. Zinc restriction resulted in higher egg output only when combined with the lowest level of dietary protein (3 %). The interaction between dietary protein and time resulted in higher egg output in mice fed low (3 %) and marginal protein (7 %) on day 22, and higher egg

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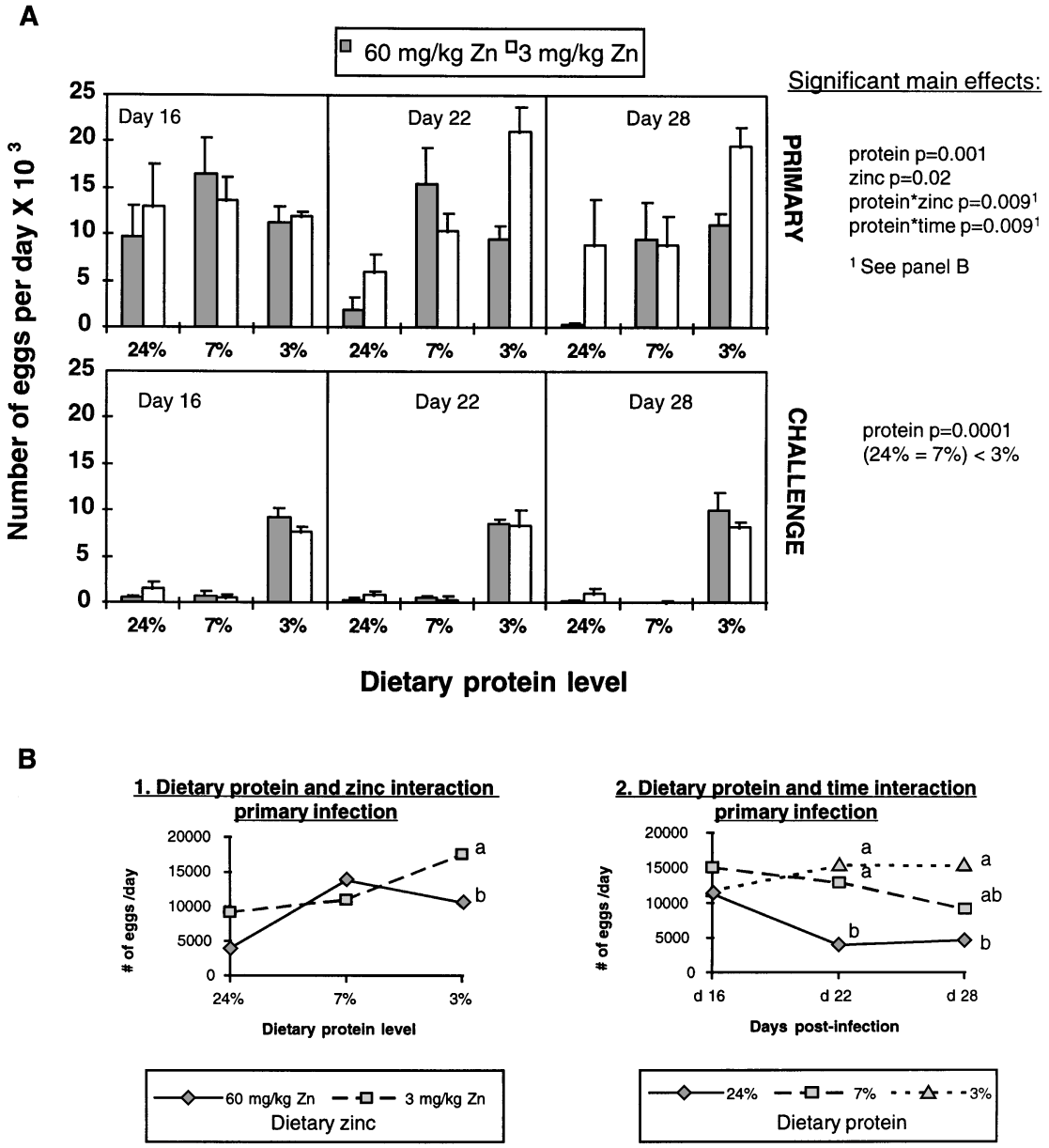

Fig. 2. The effects of dietary protein and zinc restrictions on parasite daily egg production during primary and challenge infection in mice. Values are means ± S.E.M. $N = 5$ mice/dietary treatment and infection protocol. Significant main effects of protein and zinc by day are described in (A). In (B) the significant interactions are graphically summarized. (B1) Characterization of the dietary protein–zinc interaction during a primary infection. Different lower-case letters indicate significant differences between dietary zinc levels within each protein level ($P \leqslant 0{\cdot}05$). (B2) Significant dietary protein–time interaction during a primary infection. Different lower-case letters indicate significant differences among dietary protein levels at each time-point ($P \leqslant 0{\cdot}05$).

output on day 28 in mice fed low (3%) protein than in control mice fed 24% protein. In a challenge infection (Fig. 2A), total daily parasite egg production was affected by dietary protein ($P = 0{\cdot}0001$). Mice fed 3% protein had significantly higher parasite egg output than mice fed higher protein levels throughout the period.

Eosinophils

In a primary infection (Fig. 3A), eosinophils were influenced by dietary protein ($P = 0{\cdot}002$), by dietary zinc ($P = 0{\cdot}0005$) and by time ($P = 0{\cdot}0001$). Mice fed 7% protein had higher eosinophil counts than mice fed 3 or 24% protein; mice fed 3 mg Zn/kg diet had lower eosinophil counts than mice fed 60 mg Zn/kg diet; eosinophil counts increased significantly at each time-point in all dietary groups. In a challenge infection (Fig. 3A), eosinophils were affected by dietary protein ($P = 0{\cdot}0005$), time ($P = 0{\cdot}001$) and by zinc and time interaction ($P = 0{\cdot}03$). Mice fed 3% protein had significantly lower eosinophil counts than mice fed higher protein levels. The interaction of zinc and time is shown in Fig. 3B.

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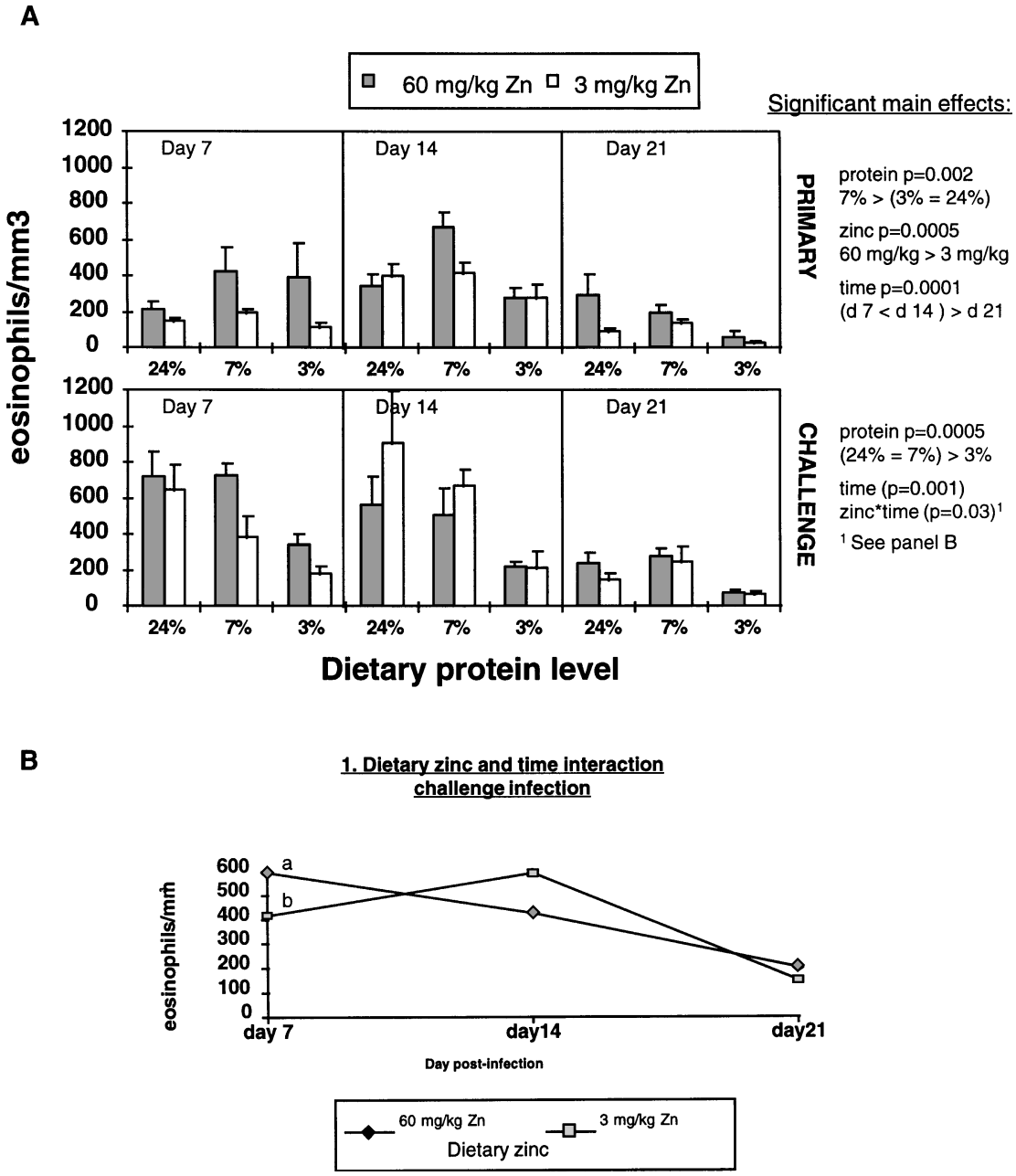

Fig. 3. The effects of dietary protein and zinc restrictions on eosinophilia during primary and challenge infection in mice. Values are means ± S.E.M. $N = 4$–5 mice/dietary treatment and infection protocol. (A) Diet and time significant main effects ($P \leqslant 0{\cdot}05$) in a primary infection: 17 % protein > (3 % protein = 24 % protein); 60 mg/kg zinc > 3 mg/kg zinc; day 7 < day 14 < day 21. In a challenge infection: (24 % protein = 7 % protein) > 3 % protein. (B) Significant dietary zinc–time interaction in a primary infection. Different lower-case letters indicate significant differences between dietary zinc levels at each time-point ($P \leqslant 0{\cdot}05$).

Mice fed 3 mg Zn/kg diet had lower eosinophil counts on day 7 post-infection, but eosinophilia was comparable between zinc treatments for the remainder of the period.

Serum total IgG1

In a primary infection (Fig. 4A) serum total IgG1 concentration was altered by dietary protein ($P = 0{\cdot}0005$), by time ($P = 0{\cdot}0001$) and by protein and time interaction ($P = 0{\cdot}006$). The nature of the interaction is shown in Fig. 4B. Mice fed 3 % protein had significantly lower total IgG1 concentration than mice fed higher protein levels on day 14 post-infection. On day 28 post-infection mice fed 3 % protein had lower total IgG1 concentration than mice fed 24 % protein only. In a challenge infection (Fig. 4A), serum total IgG1 concentration was affected by dietary protein ($P = 0{\cdot}0001$) and by time ($P = 0{\cdot}0001$). Mice fed 3 % protein had lower IgG1 concentration than mice fed 7 or 24 % protein; mice fed 7 % protein were intermediate and significantly different from the other protein levels. The serum concentration of total IgG1 increased signifi-

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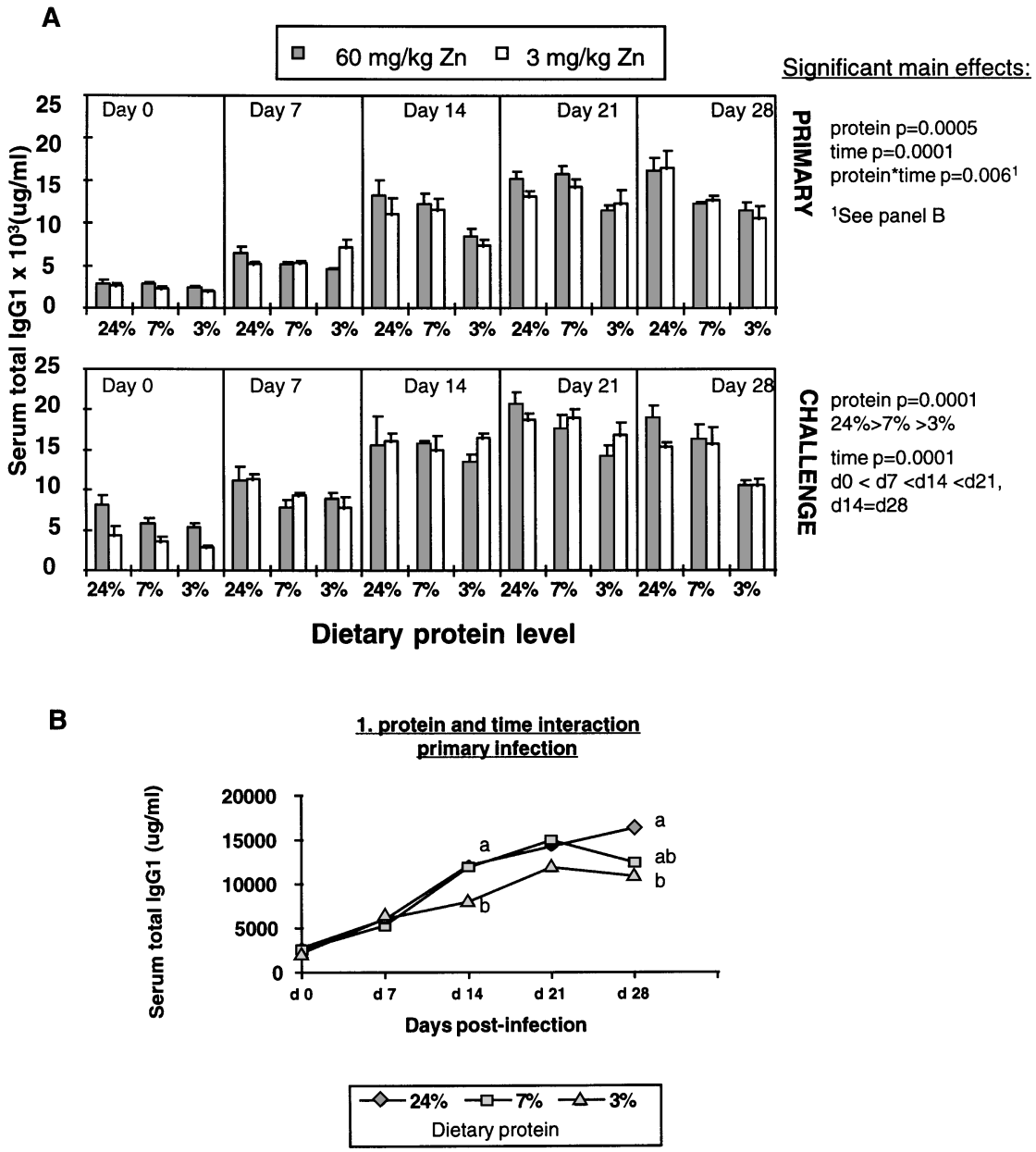

Fig. 4. The effects of dietary protein and zinc restrictions on total serum IgG1 concentration during primary and challenge infection in mice. Values are means ± S.E.M. $N = 5$ mice/dietary treatment and infection protocol. (A) Summary of significant main effects ($P \leqslant 0{\cdot}05$) in both a primary and challenge infection. (B) Significant dietary protein–time interaction in a primary infection is described. The different lower-case letters indicate significant differences between dietary protein levels at each time-point ($P \leqslant 0{\cdot}05$).

cantly with time with the exception that IgG1 concentration was not different between day 14 and day 28.

Serum parasite specific IgG1

In a primary infection (Fig. 5A) serum parasite specific IgG1 OD was affected by dietary zinc ($P = 0{\cdot}03$), by time ($P = 0{\cdot}001$), and by zinc–time interaction ($P = 0{\cdot}006$). The interaction is shown in Fig. 5B. Serum parasite-specific IgG1 OD was significantly lower in mice fed 3 mg Zn/kg diet than in mice fed 60 mg Zn/kg diet on day 14 and day 21 post-infection. In a challenge infection (Fig. 5A), serum parasite-specific IgG1 was affected by dietary zinc ($P = 0{\cdot}008$) and by time ($P = 0{\cdot}0001$). Mice fed the marginal zinc diet had significantly higher parasite-specific IgG1 than mice fed the control zinc diet. Also parasite-specific IgG1 was significantly lower on day 0 than on day 7, and it was significantly lower on those days than on day 14 and day 21, which did not differ.

Serum IgE

In the primary infection (Fig. 6A) serum IgE was affected by a protein and time interaction ($P =$

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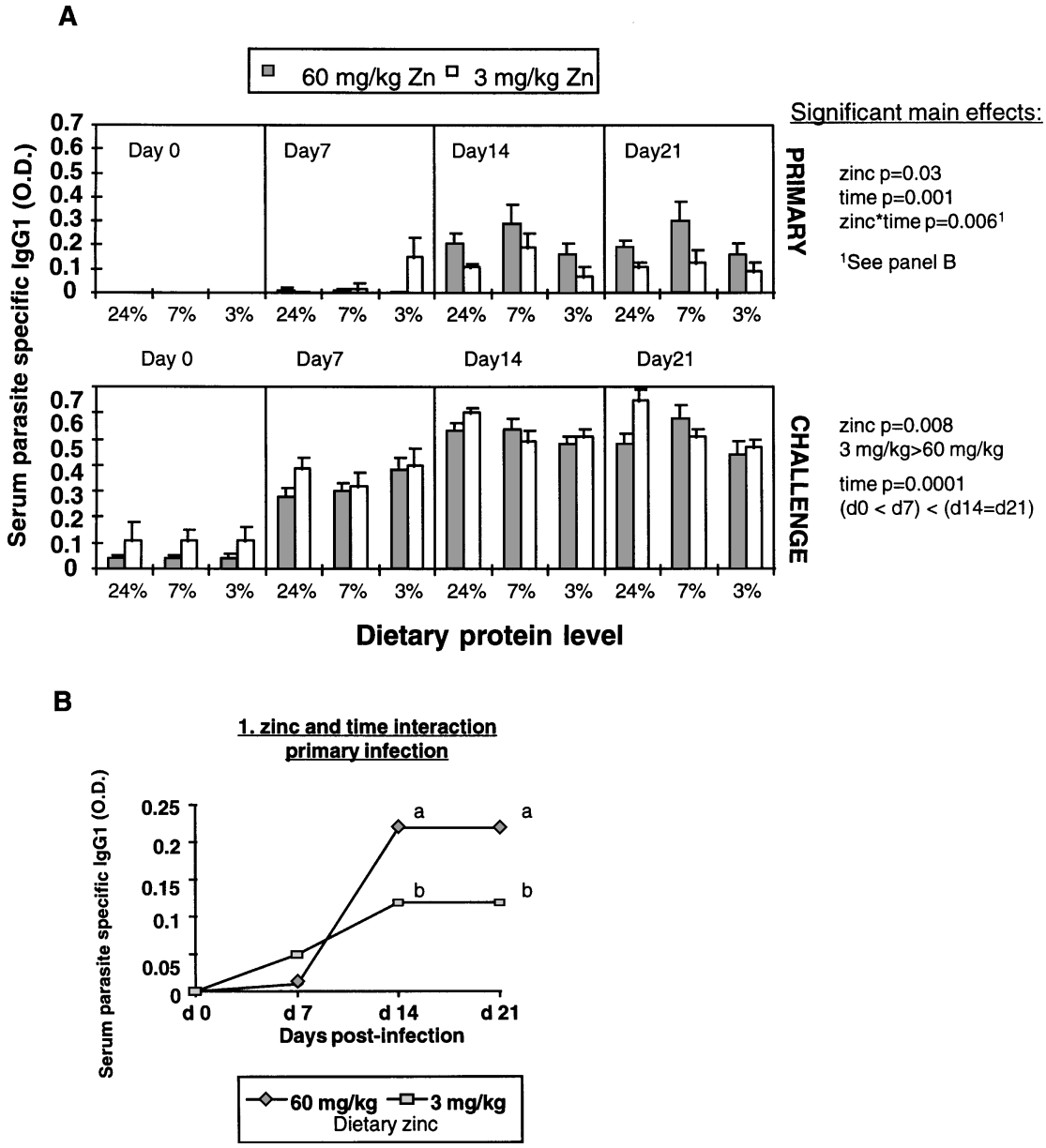

Fig. 5. The effects of dietary protein and zinc restrictions on serum parasite specific IgG1 optical density (OD) during primary and challenge infection in mice. Values are means ± S.E.M. $N = 3$–5 mice/treatment and infection protocol. (A) Significant main effects ($P \leqslant 0{\cdot}05$) in a primary infection [60 mg/kg zinc > 3 mg/kg zinc; (day 0 = day 7) < (day 14 = day 21)] and in a challenge infection [60 mg/kg zinc < 3 mg/kg zinc; day 0 < day 7 < (day 14 = day 21]. (B) Characterization of the significant dietary zinc–time interaction during a primary infection. Different lower-case letters indicate significant differences between dietary zinc levels at each time-point ($P \leqslant 0{\cdot}05$).

0·001) and by a zinc and time interaction ($P = 0{\cdot}03$). Serum IgE levels in mice fed 3% protein were higher at day 7 than in mice fed 24 and 7% protein. The same pattern is seen in the mice fed 3 mg/kg zinc such that at day 7, serum IgE levels are higher in mice fed 3 mg/kg than mice fed 60 mg/kg. Fig. 6B shows graphically the interaction of the dietary protein and zinc restriction with time. In summary, serum IgE levels increased earlier and declined in the zinc restricted, and in the most protein restricted groups, whereas IgE levels in mice fed the higher levels of protein and zinc peaked 1 week later. In the challenge infection (Fig. 6A), serum IgE increased during the first 2 weeks and declined at 3 weeks post-infection ($P = 0{\cdot}0001$). Dietary restrictions had no significant effect on serum IgE.

DISCUSSION

This was the first investigation to study the effects of a combined protein and zinc restriction on the intestinal nematode *H. polygyrus* in both naive and immunized hosts. The hypothesis that combined dietary protein and zinc restrictions would produce synergistic effects on the final outcome of infection was verified only by our results that zinc restriction

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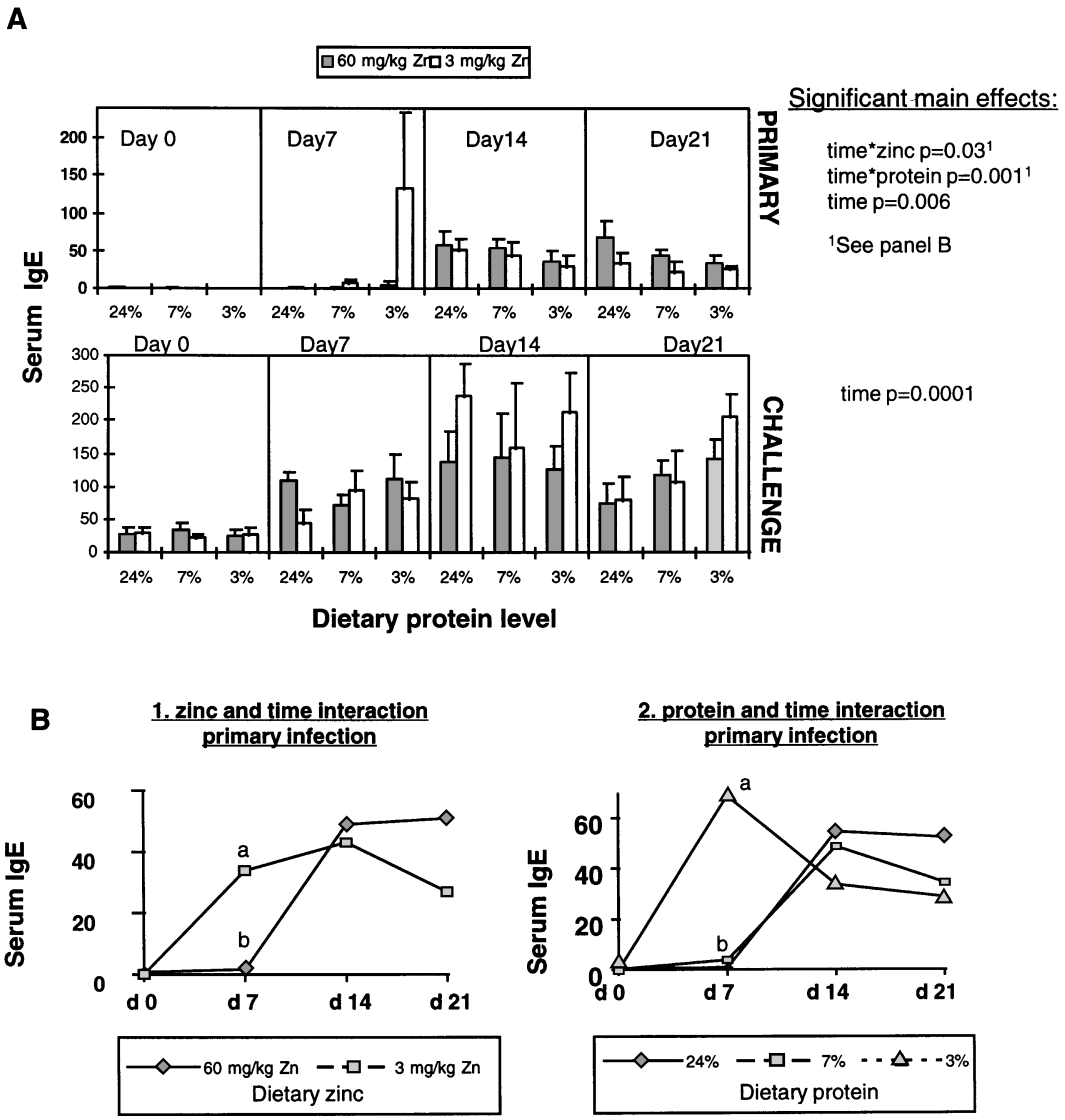

Fig. 6. The effects of dietary protein and zinc restrictions on serum IgE concentration during primary and challenge infection in mice. Values are means ± S.E.M. $N = 5$ mice/dietary treatment and infection protocol. (A) Significant main effects of time ($P < 0{\cdot}05$) for the primary and challenge infection protocols. (B) Significant zinc–time interaction ($P = 0{\cdot}03$) and protein–time interaction are characterized graphically. Different lower-case letters indicate significant differences for the specified nutrient at the designated time-point ($P < 0{\cdot}05$).

produced higher egg output only when combined with the lowest level of protein. The more general observation was that combined dietary protein and zinc restrictions exerted largely independent effects on the parasite and on the host immune response to infection and suggested that the immune responsiveness to *H. polygyrus* infection was more sensitive in immunized hosts to dietary protein than to zinc restriction. Marginal (7%) and low (3%) protein diets, and zinc restriction (3 mg/kg) resulted in prolonged parasite survival in a primary infection, whereas only the lowest level of dietary protein (3%) enhanced parasite survival during a challenge infection. A previous study on protein deficiency during a primary infection had revealed no effect of 2% protein diet on *H. polygyrus* establishment or survival in outbred CD1 mice where the primary infection is typically chronic (Brailsford & Mapes, 1987). In all instances protocols that stimulate protective immunity in the well-nourished host, and where repeated or challenge infection protocols have been used (Slater & Keymer, 1986*a*; Keymer & Tarlton, 1991), protein deficiency has been reported to prolong *H. polygyrus* survival.

Interestingly, the low zinc (3 mg/Zn/kg diet) in this study was sufficiently restrictive to enhance parasite survival during the primary infection. Therefore, the fact that this zinc intake (3 mg Zn/diet) produced higher worm burdens during a primary infection protocol and not during the challenge infection protocol in our study would strongly suggest that zinc influences the progress of infection differently in immunized (challenge) and non-immunized (primary) hosts. Previously, Minkus *et al.* (1992) found that dietary zinc restriction (5 mg zinc/kg diet) did not impair host

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resistance to *H. polygyrus* during a primary or challenge infection while Shi *et al.* (1995) observed that severely zinc deficient mice (0·75 mg zinc/kg diet) were unable to limit both a primary and challenge infection with *H. polygyrus*. Together these results indicate that low-marginal zinc restriction (3–5 mg/kg), which caused reduced tibia and liver zinc concentrations (this study) and reduced plasma zinc concentration (Minkus *et al.* 1992) but which supported normal growth (Minkus *et al.* 1992; this study), was not sufficient to alter the host immune response during a challenge infection. However, when the deficiency progressed to a more severe state, as indicated by impaired weight gain and anorexia (Shi *et al.* 1995), the ability of the zinc-deficient host to mount a protective immune response during a challenge infection was impaired.

During the challenge infection, the lower worm numbers in the marginal (7 % protein) and control (24 % protein) mice, compared with numbers in the comparable primary infection groups, confirm previous reports that the challenge infection protocol elicits a strong protective response (Behnke & Robinson, 1985). Based on worm numbers, a similarly strong protective response occurred in mice fed the low (3 mg/kg) zinc diet. In contrast, no protection was evident in the protein deficient (3 %) mice whose worm burdens 29 days after challenge infection were comparable to mice with a primary infection, suggesting that the protein deficiency impaired the development of protective immunity against this nematode. The higher intensity of infection in the low protein challenged mice correlates with our finding that eosinophil numbers remained low in mice fed 3 % protein throughout the challenge infection protocol, providing evidence of compromised immune function. This is consistent with the significantly delayed and reduced eosinophilia in mice fed a 2 % protein diet compared to mice fed a 16 % protein diet during a challenge *H. polygyrus* infection, reported by Slater & Keymer (1988). Although our 3 % dietary protein restriction did not affect the relative weights of thymus and spleen and was not sufficiently severe to produce wasting, oedema or hypoalbuminaemia, it was sufficiently severe to impair production of total IgG1 during both the primary and challenge infections, and of serum IgE during the primary infection. This indicates that dietary protein deficiency has a detrimental effect on certain systemic humoral effectors, even in the absence of overt atrophy of lymphoid organs.

An examination of dietary effects on specific components of the immune response provides an indication of the complexity of the interaction between nutritional status and immunity. Low protein intake reduced total IgG1 production during both primary and challenge infections but had no effect on parasite-specific IgG1 production. In contrast, dietary zinc deficiency had no effect on total IgG1 production, but altered parasite-specific IgG1 levels. The direction of the effect also differed, depending on the antibody measured, on whether the mouse was responding to a primary or challenge infection, and on the severity of the dietary deficiency. For example, protein deficiency resulted in elevated IgE production but reduced total IgG1 production during the primary infection. In zinc-deficient mice (3 mg/kg), parasite-specific IgG1 production was reduced during the primary infection but elevated during the challenge infection. These distinctions between the effects of protein and zinc restriction during primary and challenge infections suggest that nutritional insults influence the progress of the infection through different mechanisms.

The effects of both protein and zinc deficiency during the primary infection are of interest because there is growing evidence that, unlike many strains of mouse, BALB/c mice can mount a partially effective immune response during a primary infection (Robinson *et al.* 1989; Shi *et al.* 1994). Support for this is seen in the low recovery of worms in the control diet group (24 % protein combined with 60 mg Zn/kg diet). However, our data do not support an individual role for total IgG1, parasite-specific IgG1, IgE or eosinophilia in limiting a primary infection since there was no consistent association between increased worm survival and significantly altered immunological responses. It is possible that dietary protein and zinc restriction in a primary infection may have interfered with other more diverse components of enteric physiology such as goblet and mast cells, epithelial function and smooth muscle cell responses or other factors which may also contribute to resistance to intestinal helminths (Castro, 1989), or with sensitivity of the host to immunomodulatory products released by the worms themselves (Behnke, Barnard & Wakelin, 1992).

In conclusion, results from this study indicate that protein deficiency, induced by feeding diets containing 3 % protein, had a prominent effect in both a primary and challenge *H. polygyrus* infection. Although a dietary restriction of protein (7 %) and of zinc (3 mg/kg) can induce changes in parasite survival during a primary infection, there was no subsequent effect of these marginal restrictions on the success of the host response to a challenge infection. Our results clearly showed that combined dietary protein and zinc restriction independently affected the final outcome of infection. The maximum effects on worm survival were observed at a level of 3 % protein regardless of dietary zinc level. Most importantly, our results indicate that *H. polygyrus* is sensitive to dietary deficits and that increased parasite survival does not require profoundly compromised host nutritional status. This may be relevant to human populations where

intestinal parasitic infections and mild protein malnutrition often co-occur in the same individuals (Pawlowski, 1984). Our studies in mice suggest that dietary protein restriction in humans may predispose individuals to acquire higher levels of infection, but that marginal zinc deficiency may not further exacerbate this increased susceptibility.

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